REMARKS

A. Disposition of Claims

Claims 15-17, 19, and 23-25 are pending in this application. The obviousness rejection is appreciated as being withdrawn in view of Dr. Merril's Rule 132 Declaration dated Aug 14, 2006. Reexamination and reconsideration of the application, as amended, are respectfully requested.

B. Compliance with 35 USC 112/1 - Enablement

The Patent Office rejected the claims under 35 USC 112/1 as failing to meet the enablement requirement on the reasoning that, while the Office agrees with Applicant that one skilled in the art could attach PEG to a phage, there is no evidence that the PEGylated phage would possess the claimed property – i.e., delayed inactivation by the host defense system. (Office Action mailed 11/28/2006 at page 6, first sentence.) The rejection is respectfully traversed. The priority date of this application is April 5, 1994.

The invention solves the problem in the prior art of the use of bacteriophage to fight infections caused by bacteria. One explanation for bacteriophage not always working was because the viruses were inactivated by the host defense system. To solve this problem, the inventors proposed a technology to produce bacteriophage that may be physico-chemically altered by PEGylation to delay inactivation by the host defense system.

As claimed in Claim 19, one method of producing such bacteriophage is:

A method of obtaining a physico-chemically altered bacteriophage that is able to delay inactivation by an animal's host defense system against foreign bodies, comprising the steps of:

(a) protecting tail proteins on a bacteriophage, and

(b) then binding a polymer to any unprotected proteins on said bacteriophage, wherein said polymer is polyethylene glycol (PEG).

Starting with step (b), the Office agrees with Applicant that one skilled in the art could attach PEG to a phage.

Turning to step (a), monoclonal antibodies directed against a phage tail and that prevented phage infection were published as DeHaard et al., J Bacteriol 187: 4531 (2005), attached, and prepared using the biopanning method (at p. 4532, 2nd ¶) published in 1991:

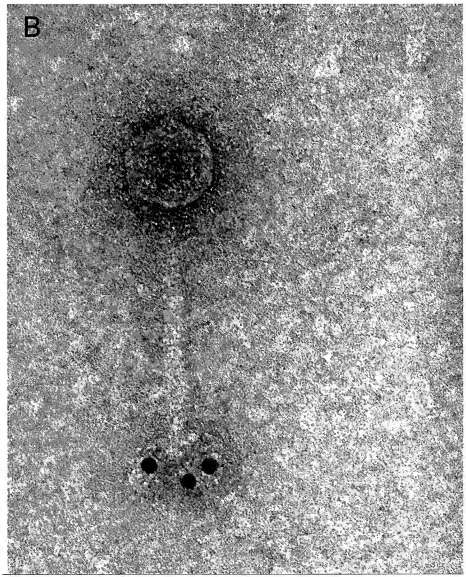
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Fig. 4B from DeHaard et al. 2005 is an immunoelectron micrograph illustrating that the immunogold labeled-antibodies recognize the tail. Additionally, monoclonal antibodies directed against a phage tail and that prevented phage infection were published as Friguet et al., J Biol Chem 265: 10347 (1990), attached, and prepared using the Kohler and Milstein technique. Thus at the time of the April 5, 1994 filing date, monoclonal antibodies directed against a phage tail, as evidenced by prevention of phage infection, could have been prepared using the biopanning method published in 1991 or the Kohler and Milstein technique.

Ending with the claimed property – i.e., delayed inactivation by the host defense system: this further step is not antithetical to patentability, because routine passaging in mice to test for PEGylated phage able to remain in the circulatory system for longer periods of time can be required without violating the enablement requirement, as described in Example 3 of the patent specification.

As for O'Riordan et al. Hum Gene Ther 10: 1349 (May 1999), of record, a time-course comparison of adenovirus numbers between PEGylated and non-PEGylated adenoviruses in the mouse model was actually illustrated in Fig. 5 and demonstrated that PEGylation of adenovirus versus sham-treated adenovirus resulted in retention of infectivity and protection from neutralizing antibody in vivo.

Likewise, covalent attachment of PEG to the surface of adenovirus by coupling PEG with tresyl-MPEG called TMPEG was reported to have preserved infectivity while reducing antigenicity. While it is true that use of other coupling methods decreased the infectivity of adenovirus (i.e., coupling PEG with cyanuric chloride-activated MPEG called "CC-MPEG" and coupling PEG with succinimidyl succinate activated MPEG called "SS-MPEG"), Applicant's preferred and exemplified method in Example 1 coupled PEG with succinimidyl carbonate activated MPEG termed "SC-MPEG". Applicant's preferred and exemplified method using SC-MPEG was not tested by the O'Riordan investigators. Additionally, the PEGylation method using TMPEG was published as Delgado et al., Biotechnol Appl Biochem 12: 119 (1990), of record. In short, not only did the O'Riordan paper not disparage Applicant's preferred and exemplified method using SC-MPEG but also it illustrated that PEGylated phage could have been prepared using O'Riordan's favored TMPEG method because it was published in 1990.

While it is true that one would have to experiment to determine which of various chemically activated forms of PEG would be compatible with protection from antigenicity and retention of infectivity, any such experimentation would not be undue given the considerable direction and guidance in the specification (e.g., Example 1, Example 3, etc.), the high level of skill in the art at the time the application was filed (the level of skill in the molecular biology art was that of a postdoctoral fellow working in the laboratory, thus the level of skill in the art was high, under *Amgen Inc. v. Hoechst Marion Roussel Inc.*, 57 USPQ2d 1449, 1518 (D. Mass. 2001)), and all of the methods needed to practice the invention were well known (i.e., monoclonal antibody techniques, PEGylation, and chemically activating PEG).

Per MPEP 2164.01(a), the In re Wands Court held that the specification was enabling with respect to the claims at issue and found that "there was considerable direction and guidance" in the specification; there was "a high level of skill in the art at the time the application was filed;" and "all of the methods needed to practice the invention were well known." Similarly, here, as indicated above, there was considerable direction and guidance in the specification; there was a high level of skill in the art at the time the application was filed; and all of the methods needed to practice the invention were well known. Considering all the factors related to the enablement issue, it must be concluded that it would not require undue experimentation to make and use the subject matter defined in the claims. The conclusion is the claims are in compliance with 35 USC 112/1 as meeting the enablement requirement.

C. Compliance With Rules Governing Information Disclosure Statements

The issue is whether the Information Disclosure Statement (IDS) mailed September 1, 2006 was considered. The rule according to MPEP 609.05 (b) is that the initials of the examiner placed adjacent to the citations on the PTO/SB/08 (or equivalent) form mean that the information has been considered by the examiner. Here, Applicant has not received an indication that the information was considered. Applicant respectfully requests that the initials of the examiner be placed adjacent to the citations on the IDS mailed September 1, 2006 to indicate that the information has been considered by the examiner and a copy mailed to Applicant.

CONCLUSION

Applicant respectfully requests that a timely Notice of Allowance be issued in this case. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the below-given telephone number.

Respectfully submitted,

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Dated: 2/28/07

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JOURNAL OF BACTERIOLOGY, July 2005, p. 4531–4541 Vol. 187, No. 13
0021-9193/05/$08.00+0 doi:10.1128/JB.187.13.4531–4541.2005

Llama Antibodies against a Lactococcal Protein Located at the Tip of the Phage Tail Prevent Phage Infection

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Received 9 September 2003/Accepted 7 October 2004

Bacteriophage p2 belongs to the most prevalent lactococcal phage group (936) responsible for considerable losses in industrial production of cheese. Immunization of a llama with bacteriophage p2 led to higher titers of neutralizing heavy-chain antibodies (i.e., devoid of light chains) than of the classical type of immunoglobulins. A panel of p2-specific single-domain antibody fragments was obtained using phage display technology, from which a group of potent neutralizing antibodies were identified. The antigen bound by these antibodies was identified as a protein with a molecular mass of 30 kDa, homologous to open reading frame 18 (ORF18) of phage sk1, another 936-like phage for which the complete genomic sequence is available. By the use of immunoelectron microscopy, the protein is located at the tip of the tail of the phage particle. The addition of purified ORF18 protein to a bacterial culture suppressed phage infection. This result and the inhibition of cell lysis by anti-ORF18 protein antibodies support the conclusion that the ORF18 protein plays a crucial role in the interaction of bacteriophage p2 with the surface receptors of *Lactococcus lactis*.

Lactococcus lactis is a gram-positive lactic acid bacterium used for the manufacture of fermented dairy products (2). The milk fermentation process is susceptible to infection by bacteriophages found in raw milk (3, 19, 32–34) or by induction of prophages from lysogenic starter strains (19). The phage infection results in lysis of the bacteria, leading to production delays, variations in the taste and texture of the products, or even complete failure of fermentation. To minimize economic losses by phage infections, a variety of precautions are used (35, 36). Lactococcal phages fall into three prevalent groups of DNA homology, 936-, c2- and P335-like phages (32–34). Characteristics of these phages include a double-stranded DNA genome and a long noncontractile tail. The 936 and P335 groups have a small isometric head, while members of the c2 group have a prolate head.

We describe here the generation of phage-neutralizing monoclonal single-domain antibody fragments (V_HH) derived from cameloid heavy-chain antibodies. In the blood of *Camelidae*, a high proportion of the immunoglobulins consists of homodimers of only heavy chains, devoid of light chains (17). As described in this and other papers, it is possible to elicit good immune response in camelids against complex protein mixtures, phages, or even whole organisms (26). Genes encoding V_HH fragments that bind to these complex protein mixtures can be selected easily. In such libraries of binders, there is a high probability of finding V_HHs that block essential biological processes, mainly because of the long CDR3, which can block active centers (27).

It was demonstrated that after immunization of a llama with lactococcal bacteriophage p2 (936 group) the fraction of heavy-chain antibodies contained about 10-fold higher neutralizing activity than conventional antibodies. We generated a phage display library (31, 39) from which binding and neutralizing single-domain fragments were selected. Nanomolar concentrations of one of these V_HHs efficiently neutralized lactococcal bacteriophage p2 even in milk fermentation on a semi-industrial scale (28). Here we show that the antigen open reading frame 18 (ORF18) turned out to be a structural protein, and it was demonstrated that this protein is located at the tip of the phage tail. The methods developed and the knowledge generated by this study will lead ultimately to a better understanding of the molecular mechanism of phage-host interactions and to new effective ways to prevent viral infections by application of llama antibody fragments.

MATERIALS AND METHODS

Selection and screening of lactococcal bacteriophage-specific single-domain antibody fragments from a llama immune phage display library. Phage p2 was purified, amplified, and concentrated as described previously (1). A llama was immunized at days 0, 30, 58, and 86 with 3×10^9 PFU of *L. lactis* bacteriophage p2 as described previously (10). The immune response was followed by titration of serum samples in an enzyme-linked immunosorbent assay (ELISA) with phage p2 coated at a titer of 10^{10} PFU/ml in phosphate-buffered saline (PBS) following the protocol described before (10).

Peripheral blood lymphocytes were isolated from a 150-ml blood sample, taken 7 days after the last immunization, via a Ficoll-Paque gradient yielding about 10^8 blood cells and comprising about 10^7 B cells. Total RNA (between 250 and 400 μg) was extracted (5) and used for the preparation of random primed cDNA (8), which served as the template for amplification of the V_HH genes with oligonucleotide primers V_HH-2B, Lam-07 (priming to the short hinge region), and

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Lam-08 (long hinge specific) (10, 45). PCR was performed as described by De Haard and colleagues (8).

The amplified products were digested with PstI and NotI and cloned in phagemid vector pUR5068, which is identical to pHEN1 (21) but contains a hexahistidine tail for immobilized metal affinity chromatography (20) and a c-*myc*-derived tag for detection. Ligation and transformation were performed as described previously (8).

The rescue with helper phage VCS-M13 and polyethylene glycol precipitation was performed as described previously (30). Selections were done via the biopanning method (30) by coating of phage p2 (10^{16} PFU/ml at round 1 and 10^{9} PFU/ml at round 2) or via the in-solution seleetion method (18, 46) with in vitro biotinylated phage (3×10^{13} PFU/ml at the first round and 3×10^{12} PFU/ml at the second round).

Soluble $V_{H}H$ was produced by individual clones as described previously (30). Culture supernatants were tested in ELISA using immobilized phage p2; bound $V_{H}H$ was detected with a mixture of the mouse anti-myc monoclonal antibody 9E10 (500 ng/ml) and anti-mouse horseradish peroxidase conjugate (DAKO; 4,000-fold diluted). Fingerprint analysis (46) with the restriction enzyme HinFI (New England Biolabs) was performed on all clones.

Production of soluble $V_{H}H$ fragments by inducing 50-ml cultures and preparation of periplasmic fractions, which were used for ELISA experiments, were done as described previously (8). DNA sequencing was performed at Baseclear B.V. (Leiden, The Netherlands).

Production of $V_{H}H$ fragments in *Escherichia coli* and *Saccharomyces cerevisiae*. For large-scale production (400 ml) in *E. coli*, the $V_{H}H$-encoding gene fragments were recloned via PstI/BstEII digestion in vector pUR5850 (Fig. 1A). This vector is identical to phagemid vector pUR5068 but lacks gene 3 and contains an additional carboxy-terminally located tag sequence of 15 amino acids, which encodes an in vivo biotinylation signal (41). Alternatively, an *E. coli* production vector was used encoding a different peptide sequence of five amino aeid residues (TAG) recognized by a monoclonal antibody instead of the c-*myc* tag.

After induction of $V_{H}H$ gene expression (8), a soluble protein fraction was prepared by disruption of cells with a French press using a volume of 3.5 ml of cell suspension in 0.1 mol/liter phosphate buffer (pH 7) in an FA-003 minicell at 20,000 lb/in^2 (American Instrument Company). This process was followed by removal of the insoluble proteins via high-speed centrifugation (30 min at 13,000 × *g* at 4°C). The antibody fragments were purified from the lysate via their hexahistidine tail using Talon column material (Clontech).

For secretion by *S. cerevisiae*, the fragments were recloned in episomal vector pUR4547 (Fig. 1B), which is identical to previously described pUR4548 (10) but does not encode any tag sequences. The host strain used, VWK18ga11, was a *gal1* derivative of CEN.PK102-3A (*MATa leu2 ura3*) obtained by disruption of the *GAL1* gene by integration of the *S. cerevisiae URA3* gene (40) at this locus. Productions on a 0.5-liter (shake flasks) or a 10-liter scale was performed at BAC B.V. (Naarden, The Netherlands) as described previously (43). The $V_{H}H$ fragments were purified by ion-exchange chromatography with Mono-S-Sepharose (Pharmacia) after concentrating the culture supernatant by ultrafiltration. The purification yield was determined by measuring the optical density at 280 nm (OD_{280}), using the molar extinction coefficients calculated from the encoded amino acid sequence (program ProtParam-tools at www.expasy.ch). The purity was analyzed on Coomassie-stained 15% sodium dodeyl sulfate-polyacrylamide gel electrophoresis gels.

Bihead molecules were produced in *S. cerevisiae* by introduction of an XhoI site (instead of PstI) in the FR1-encoded primer and cloning of the PCR product as an XhoI/BstEII fragment in an adapted version of episomal vector pUR4547. This vector, pJS9, allows the insertion of another $V_{H}H$ gene downstream of the first one via digestion with PstI/HindIII.

The avidity of the purified $V_{H}H$ fragments was analyzed by gel filtration using a Superose 12 column combined with mass spectroscopy using matrix-assisted laser desorption ionization–time of flight mass spectrometry. In addition, a bispecificity ELISA was performed according to the protocol described above using p2 phage as coating. Detection was accomplished with a mixture of in vitro biotinylated phage p2 (at 10^{9} PFU/ml) and streptavidin-horseradish peroxidase conjugate (DAKO; 1,000-fold diluted).

Determination of neutralizing capacity with plaque titration and small- and large-scale neutralization assays. Plaque titration was performed according to Terzaghi and Sandine (42). Indicator strains *L. lactis* subsp. *cremoris* LM0230 and C2 (7, 13) were used for titration of phages p2 and sk1.

The small-scale neutralization assay was performed by culturing *L. lactis* LM0230 or C2 (1% inoculum overnight culture) in microtiter plates using 100 μl of sterilized semiskim milk containing 0.35% peptone (Difco), 0.35% yeast extract (Difco), 1% glucose, 0.5% polymyxin B (Oxoid), 1% bromophenol red (Sigma), and 10^{5} PFU/ml of phage p2 in the presence of variable concentrations

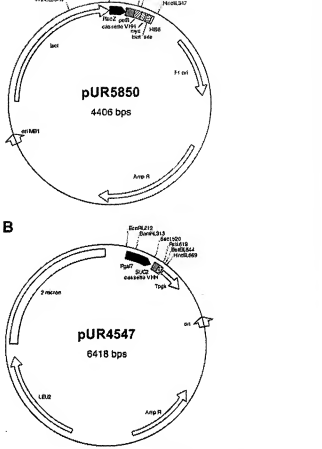

FIG. 1. Plasmids used in this study. (A) Expression vector for large-scale production of $V_{H}H$ fragments in *E. coli*. PlacZ = IPTG-inducible promoter; pelB = signal sequence; myc and HIS6 encode tags; biot = biotinylation site. (B) Expression/integration vector used to transform *S. cerevisiae* and to express the various $V_{H}H$ fragments. Pgal7 = galactose-inducible promoter, SUC2 = invertase signal sequence, Tpgk = the PGK terminator. LEU2 is the *LEU2* gene behind a defective promoter, which ensures, together with the 2 micron sequence, the multicopy integration of this vector on the rDNA locus.

of antibody fragments (in culture supernatant of *E. coli*, in llama sera, or as purified antibody fragments). After overnight incubation at 30°C, growth of the cells was visualized by the yellow color of the indicator, whereas a purple color indicated repressed growth due to lysis of the cells.

The large-scale assay was performed by culturing in 20 ml LM17 medium at 30°C containing variable titers of phage p2 and concentrations of purified $V_{H}H$ fragment or ORF18. The pH and OD_{600} of 1-ml samples were measured.

Characterization of recognized antigens. For immunoelectron microscopy, 150-mesh nickel grids were coated with Formvar, followed by carbon evaporation. The grids, with their coated side up, were glow discharged for 30 s in air at a pressure of about 0.1 torr. Thereafter, the grids were put two times for 10 s with the coated side down on a droplet of distilled water, followed by floating for 10 min on a 20-μl droplet of phage solution in 100 mM PBS (pH 7.2) with a titer of 10^{10} PFU/ml. Excess phage suspension was removed with filter paper. All incubations were performed at room temperature. Antibody fragments $V_{H}H\#2$ and $V_{H}H\#5$ were diluted in 1% bovine serum albumin in PBS (BPBS) to a concentration of about 20 μg/ml and incubated for 15 min with the grids. After five washes for 1 min by floating on droplets of BPBS, the different grids were placed, sample side down, for 15 min on a 50-μl droplet of rabbit anti-$V_{H}H$ polyclonal antibody solution (50-fold dilution in BPBS). After washing with BPBS (as

before), each grid was incubated for 15 min in a 50-μl droplet of goat anti-rabbit polyclonal immunoglobulin G antibodies conjugated to 10-nm gold particles (Aurion B.V., Wageningen, The Netherlands) diluted 10-fold in BPBS. After five washes by floating in droplets of distilled water, the labeled phages were negatively stained on a 100-μl droplet of 2% aqueous uranyl acetate solution for 1 min, followed by removal of the excess stain with filter paper. After drying at room temperature for 30 min, the labeled phages were examined with an EM420 transmission electron microscope (Philips) and micrographs were taken at an acceleration voltage of 80 kV.

Epitope mapping was performed with the lambda gt11 system using the method described by Mondelli and colleagues (37), in which random fragments were generated from the genomic DNA of phage p2 and cloned in lambda gt11 (Promega). For screening of the expression library, approximately 5×10^4 plaques per plate (14 cm diameter) were analyzed. The inserts from phage clones which bound to the V_HH were amplified with gt11 forward and gt11 reverse primers (Promega) and cloned into the pGEM-T easy vector (Promega), which subsequently was sequenced with T7 and M13 reverse primers (Promega). The epitope recognized was localized further by the pepscan method (15) using overlapping 15-mer peptides derived from the major structural protein (msp) gene product from phage sk1 as described previously (4).

For amino-terminal sequencing, 10^{11} phage p2 particles were loaded on a 15% polyacrylamide electrophoresis gel and blotted on a polyvinylidene difluoride membrane (ProBlott; Perkin-Elmer). The blot was stained with Coomassie brilliant blue, the band of interest was excised, and the amino-terminal sequence determined with an LF-3000 Protein Sequencer (Beckman). The individual phenylthiohydantoin amino acid derivatives from the Edman degradation were monitored online and analyzed with a System Gold high-performance liquid chromatography system (Beckman).

Purification of phage p2-encoded gene products expressed in *E. coli*. All the primers used were based on the genomic sequence of sk1 (4). The gene encoding the major structural protein (msp; ORF11) was amplified from phage p2 with msp 5' primer 5'-GTC CTC GCA ACT GCC GTC TCC CAT GAA ATT AGA TTA TAA TTC ACG TGA GAT-3' and msp 3' primer 5'-GAG TCA TTC TCG ACT TGC GGC CGC TGA ATG GTC AGT TAC TGA AAC TCC TGC GGT-3' using AmpliTaq Gold (Perkin-Elmer). The lysin gene (ORF20) was obtained by amplification with lys 5' primer 5'-GTC CTC GCA ACT GCC GTC TCC CAT GAA TAT AAC TAA TGC TGG CGT-3' and lys 3' primer 5'-GAG TCA TTC TCG ACT TGC GGC CGC TTT TTT AGC AAT GAT TGG TTT GT-3'. Finally, ORF18 was amplified with ORF18 5' primer 5'-GTC CTC GCA ACT GCC GTC TCC CAT GAC AAT TAA AAA GTT CAC GTT TTT CA-3' and ORF18 3' primer 5'-GAG TCA TTC TCG ACT TGC GGC CGC TTT AAT GAA GTA ACT TCC GTT ACC-3'. All 5'-end primers contain a BsmBI site (shown in bold characters), which enables cloning in the NcoI site of vector pET28a (Novagen), thereby creating the ATG start codon. The 3'-end primers have a NotI site (also in bold), which gives an in-frame fusion to the hexahistidine tail when ligated to the corresponding site of pET28a. The PCR products were purified from gel, digested with BsmBI and NotI, and after spin dialysis against water, ligated to NcoI/NotI-digested pET28a and electroporated into *E. coli* BL21-CodonPlus(DE3) cells (Novagen).

The ORF18-containing constructs were made from two individual PCR products and sequenced with the T7 and T7rev primers at BaseClear B.V. (Leiden, The Netherlands), thereby excluding errors introduced by amplification.

For production of the protein, transformants were grown at 37°C in 400 ml 2TY medium containing kanamycin (100 μg/ml) until a late-log-phase culture was obtained (OD_{600}, approximately 0.9). The culture was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM), and growth was continued for 4 h. Cells were harvested and disrupted in a French press as described previously. The hexahistidine-tagged proteins were purified from the soluble protein fraction with Talon (Clontech). Purity was analyzed on Coomassie-stained polyacrylamide electrophoresis gels.

Affinity measurements with surface plasmon resonance. Binding kinetics were analyzed by surface plasmon resonance on a Biacore-3000 (Biacore) using a surface-containing anti-TAG monoclonal antibody (approximately 9,000 response units) covalently coupled to a CM5 chip (Biacore). A fixed amount of tagged antibody fragment V_HH#5 was captured (120 and 300 response units), and a variable concentration of purified ORF18 (between 30 nM and 5 μM) was injected at a flow rate of 10 μl/min. For an accurate determination of the off rate, the low-density surfaces were used to avoid rebinding as could be concluded from the monophasic dissociation. The on rate was determined from those measurements, which showed no mass transport limitation.

RESULTS

Isolation of lactococcal bacteriophage p2-specific single-domain antibodies via phage display. A llama was immunized with purified bacteriophage particles from *L. lactis* phage p2. The antigen-specific immune response was followed by ELISA. During these titrations, the total content of serum antibodies was measured and no discrimination was made between the response of the "classical" antibodies (i.e., containing a heavy and a light chain) and the heavy-chain antibodies. After 3 weeks, the sera contained high titers of anti-p2 antibodies.

An important objective of this study was to identify V_HH fragments capable of preventing lactococcal phage infection by monovalent binding to bacteriophage particles. In addition to analyzing individual phage-binding antibody fragments in ELISA, a phage microtiter plate neutralization assay was used that permitted the analysis of large numbers of clones.

The performance of the assay was evaluated with immune sera from the llama. The serum taken after the fourth immunization had high titers of neutralizing antibodies, since complete inhibition of infection at a phage titer of 10^5 PFU/ml was obtained even at a serum dilution of 10^{-4}. As expected, the preimmune serum did not show neutralization. The heavy-chain and classical double-chain antibodies were purified from the postimmune serum and tested in serial dilutions. The long-hinge-containing heavy-chain antibodies inhibited infection with phage titers of 10^5 PFU/ml at an antibody concentration of 620 ng/ml and the short-hinge heavy-chain antibodies at a concentration of 960 ng/ml. The classical antibodies were less efficient and gave only partial neutralization at 9.5 μg/ml. Following RNA isolation from B lymphocytes, the gene segments encoding the V_HHs were amplified and cloned to obtain a phage display library with approximately 10^7 clones. After two rounds of biopanning or selection with in vitro biotinylated *L. lactis* phage p2, an increasing number of phage clones was eluted, indicating successful selection. We used both selection methods as antibodies with different binding characteristics are selected by these methods (46). Several hundred clones sampled from the unselected library and after both rounds of selections were screened in an ELISA for the production of p2-specific V_HH fragments. A large fraction of clones bound p2 positive, increasing from 60% after round 1 to 95% after round 2, while no binding antibodies were found in 32 analyzed clones from the unselected library. Furthermore, many different phage p2-specific V_HH-encoding clones were selected from the library, as demonstrated by HinFI fingerprint analysis (details not shown).

Identification of phage p2 neutralizing antibodies with acidification screenings assay. The culture supernatants of the V_HH-producing clones were tested in phage neutralization assays, which showed that 6 out of 250 (2.4%) antibody fragments analyzed inhibited phage infection completely and 36 out of 250 (14.4%) gave some degree of neutralization. It should be noted that the most potent neutralizing V_HH fragments gave poor signals in the ELISA screening and would have been missed if only a limited number of ELISA-positive clones were analyzed with plaque-forming assays.

A panel of four nonneutralizing (V_HH#1 to V_HH#4) and three neutralizing (V_HH#5 to V_HH#7) antibody fragments with different HinFI fingerprint patterns was studied in more

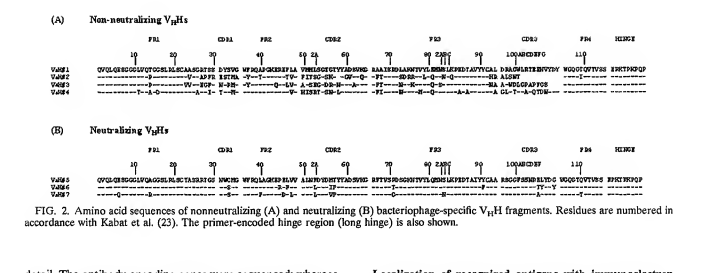

FIG. 2. Amino acid sequences of nonneutralizing (A) and neutralizing (B) bacteriophage-specific V_HH fragments. Residues are numbered in accordance with Kabat et al. (23). The primer-encoded hinge region (long hinge) is also shown.

detail. The antibody-encoding genes were sequenced; whereas the nonneutralizing V_HH fragments have divergent sequences, the neutralizing V_HH fragments were very similar, indicating that they all recognized the same epitope (Fig.2). The genes encoding these V_HH fragments were recloned in an episomal vector designed for secretion of V_HHs by *S. cerevisiae* (10, 46) (Fig. 1B). The purified antibodies were diluted and tested in the small-scale acidification assay, in the plaque formation assay, and in the ELISA. None of the four nonneutralizing antibodies prevented infection of phage p2, although they have a good antigen-binding capacity. The three neutralizing antibodies completely inhibited phage infection (data not shown).

Efficiency of neutralization of V_HH#5 and cross-reactivity against other lactococcal phage groups. The ability to neutralize phage was studied in more detail by regularly measuring the pH of small-scale cultures (20 ml). A fixed titer of phage (10^3 PFU/ml) was combined with one concentration (667 nM) of the antibody fragment. The acidification curve from the phage-infected culture containing neutralizing antibody fragment V_HH#5 was indistinguishable from the noninfected control, thus showing the normal drop of pH from 6.6 to 4.6 after 10 h of cultivation. The infected culture combined with nonneutralizing fragments V_HH#2 or V_HH#3 showed no change in pH, indicating that most lactococcal cells were lysed as a consequence of phage infection (data not shown). Antibody fragment V_HH#5 prevented phage infection, even at concentrations as low as 2.25 nM, as can be seen from the acidification and the cell density profiles of the cultures (Fig. 3A). Higher phage titers ($>10^5$ PFU/ml) could also be neutralized with higher concentrations of antibody (Fig. 3B).

The cross-reactivity against other lactococcal phages was investigated with the microtiter plate acidification assay. Phage sk1, a 936-like phage, was neutralized by V_HH#5 as efficiently as phage p2. Three nanomolar V_HH#5 completely protected the *L. lactis* host cells against phage infection, whereas a 10-fold lower concentration (0.3 nM) failed to do so. Preimmune llama serum showed no inhibition, while postimmune serum also gave identical inhibition profiles for phages p2 and sk1 (phage neutralization at a serum dilution of 10^{-3}, no inhibition at 10^{-4}). However, two members of the c2 group, phages Q38 and c2, were not neutralized by V_HH#5, although postimmune llama serum slightly inhibited infection at a 10-fold dilution.

Localization of recognized antigens with immunoelectron microscopy and analysis of fine specificity by epitope mapping. Immunoelectron microscopy was performed to localize the proteins recognized by the different V_HH fragments on the phage structure. Using immunogold labeling, it was shown that both nonneutralizing antibodies V_HH#2 (Fig. 4A) and V_HH#3 (not shown) bound to proteins located on the phage capsid. In contrast, neutralizing antibody V_HH#5 recognizes a protein at the tip of the tail (Fig. 4B), which is the site involved in host recognition during the phage adsorption process.

To identify the epitopes of the antibodies, a lambda gt11 expression library was constructed containing random genomic DNA fragments from phage p2 approximately 50 to 250 bp in length (37). Screening of approximately 100,000 lambda plaques with V_HH#2 resulted in the identification of six positive clones. The inserts were subcloned, and sequencing demonstrated that the antibody recognizes the carboxy-terminal domain of the major structural protein (msp) of phage p2 (Fig. 5A). The epitope was localized further by pepscan analysis of the 79-amino-acid peptide shared by the six gt11 clones (Fig. 5B) and was shown to consists of the sequence NGQLAPGV YIVTFSA, corresponding to residues 271 to 285 of the msp. This result also demonstrated, for the first time, that the msp (ORF11) of lactococcal 936-like phages is the predominant protein of the capsid.

Upon screening of the gt11 expression library with neutralizing antibody V_HH#5, no binding clones were obtained, thereby prompting an alternative approach to identify the antigen.

Characterization of antigen recognized by neutralizing antibody by Western blot analysis and amino-terminal sequencing. To exclude a mechanism of neutralization based on avid binding of multimerized antibody fragments leading to inactivation of phage by aggregation, the valency of purified V_HH#5 was determined by gel filtration (16) and mass spectrometry (Fig. 6). Gel filtration revealed a molecular mass of 12.1 kDa and mass spectroscopy 13.5 kDa for V_HH#5, suggesting that the antibody fragment indeed has a monomeric appearance. A bihead fragment (6) containing head-to-tail-linked V_HH#3 and V_HH#2 (3-2) emerged from the column in a single peak corresponding to the dimeric product (molecular mass of 26.6 kDa as determined with mass spectroscopy; data not shown).

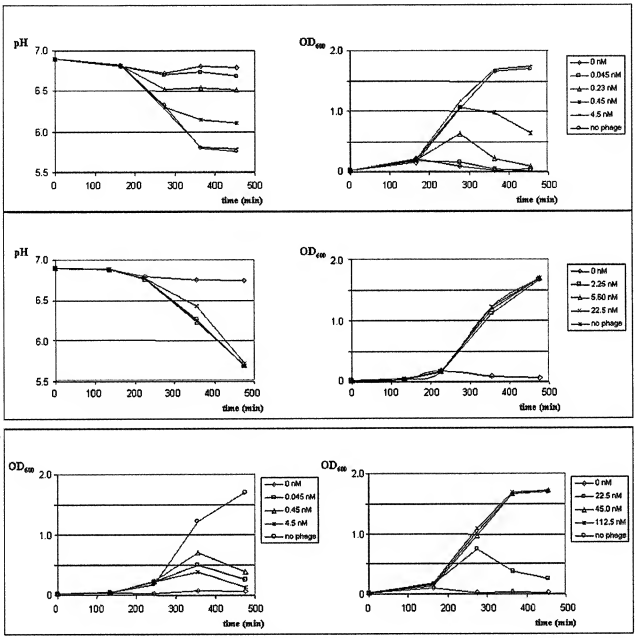

FIG. 3. Acidification profiles and growth kinetics of phage-infected cultures as a function of the concentration of $V_HH\#5$. (A) Determination of the minimal concentration of V_HH to neutralize an infection of 10^3 PFU/ml phage p2 by measuring pH and OD_{600}. (B) Growth kinetics determined with OD_{600} for a culture containing 10^5 PFU/ml phage p2 and variable amounts of V_HH.

Furthermore, a bispecificity ELISA was developed in which phage p2 was coated to capture the bivalent antibody fragment that could be detected with in vitro biotinylated p2 phages. No response was obtained with $V_HH\#5$ when detection was performed with biotinylated p2 phages, while positive signals were found upon incubation with the anti-*myc* antibody, thereby confirming the monovalent character of this antibody fragment. The bihead molecule 3-2, which was used as a positive control in this assay, gave high signals with biotinylated p2 phage or anti-*myc*. From these experiments it was concluded that the neutralizing capacity of $V_HH\#5$ is not caused by aggregation of phage particles but that the nature of the phage-derived antigen recognized by the antibody is crucial.

The structural protein recognized by neutralizing antibody fragment $V_HH\#5$ was identified by Western blot analysis (Fig. 7). Here, phage sk1 was used instead of phage p2 because its complete genomic sequence is available (4). A clear band was visible on blot after incubation with $V_HH\#5$, and comparison with a Coomassie stained blot showed that the recognized antigen migrates somewhat faster than the msp. The amino

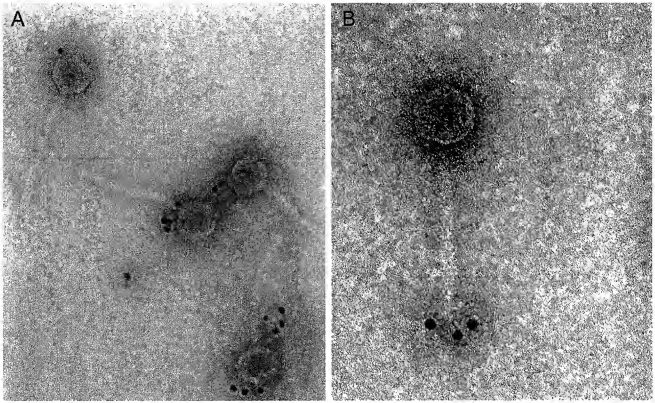

FIG. 4. Immunoelectron microscopy with heavy-chain antibody fragments and 10-nm gold-labeled detection antibodies on bacteriophage p2. (A) Phage detected with nonneutralizing $V_HH\#2$. (B) Phage recognized by neutralizing fragment $V_HH\#5$.

terminus of the structural protein recognized by $V_HH\#5$ is identical to the hypothetical gene product of ORF18 of phage sk1. The molecular mass (30 kDa), as estimated from a gel, suggests the presence of the complete ORF18 product (containing 264 amino acid residues) in the bacteriophage particle.

To confirm the observed specificity the ORF18 protein, the major structural protein (msp) (ORF11) and lysin (ORF20) expressed in *E. coli*, as well as complete phage sk1 particles, were tested in Western blot assays with $V_HH\#5$ (Fig. 8). The neutralizing antibody $V_HH\#5$ indeed recognized bacterially expressed ORF18, which comigrated with the phage-bound protein. As a control, fragment $V_HH\#2$ was included in the experiment and as expected, this antibody reacted with the mcp produced in *E. coli* and the phage-associated antigen. In contrast, anti-mcp fragment $V_HH\#3$ also reacted with the bacterially expressed msp (containing the phage p2 gene) but failed to recognize the corresponding product of phage sk1 (data not shown).

The affinity of $V_HH\#5$ to purified ORF18 was determined by analysis of the kinetics of the antibody-antigen interaction using surface plasmon resonance. The association rate k_a was $(3.49 \pm 0.51) \times 10^5\ M^{-1}\ s^{-1}$, and the dissociation rate k_d was $(4.82 \pm 1.09) \times 10^{-4}\ s^{-1}$, resulting in a K_D of $(1.40 \pm 0.21) \times 10^{-9}$ M or 1.40 nM.

Competition assays with purified ORF18. Since binding of an antibody fragment to ORF18 prevents infection, it was hypothesized that this gene product might have an important function during phage adsorption to the *L. lactis* host receptor. This hypothesis was evaluated by adding *E. coli*-produced ORF18 to phage-infected *L. lactis* cultures. We speculated that the added ORF18 would compete with the ORF18 bound to phage p2 particles for binding to the cellular receptor and thereby prevent phage infection. At relatively high concentrations of ORF18 (approximately 500 nM) and low titers of p2 phage (10^2 and 10^3 PFU/ml), phage infection of *L. lactis* cells was completely prevented, while at lower concentrations of ORF18, the phage infection process was only retarded (Fig. 9). This addition of purified mcp had no effect on phage infection (data not shown).

DISCUSSION

The vulnerability of lactic acid bacteria to phage attack still is the biggest problem associated with industrial manufacture of fermented dairy products (2). Phage p2 is a typical member of the most frequently isolated lactococcal phage group, while heavy-chain antibodies of *Camelidae* have a number of very interesting properties and applications (10, 26, 43, 44, 46). We decided to use these antibodies to develop a new powerful route to neutralize phage and simultaneously to elucidate unknown mechanisms of phage infection of lactic acid bacteria. Evaluation of the neutralizing capacity of antibodies purified from postimmune llama serum showed that the heavy-chain antibodies inhibited phage infections more efficiently than classical antibodies. Overall, these data indicate that camelid heavy-chain antibodies can eliminate viral infections efficiently,

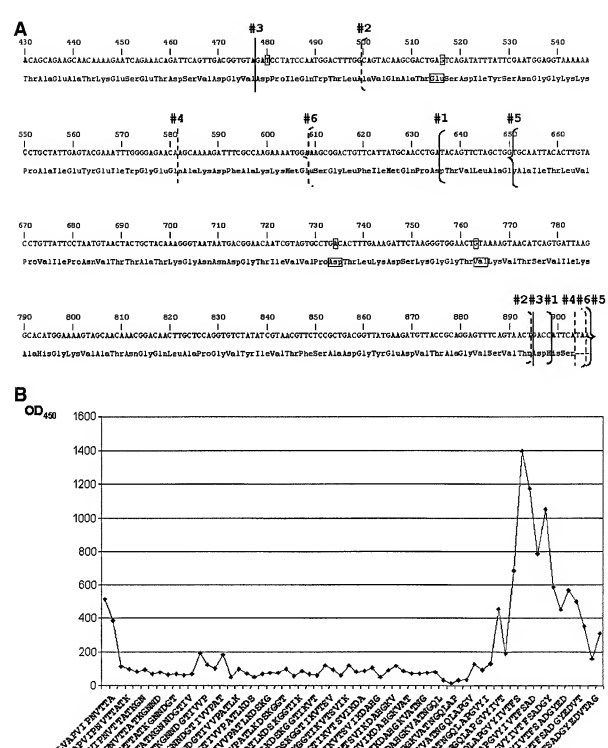

FIG. 5. Epitope mapping of anti-msp antibody fragment $V_HH#2$. (A) Inserts of the $V_HH#2$-recognized lambda gt11 clones (coded #1 to #6), which encode a shared segment of the carboxy-terminal region of the major structural protein. Numbering has been done as in the deposited sk1 genomic sequence (GenBank accession number AF011378). The earlier observed differences (25) between the shown p2-derived sequence and the sk1 sequence are indicated with boxes. (B) Pepscan results obtained with overlapping 15-mer peptides based on the shared sequence segment of the lambda gt11 clones. The peak corresponds with the peptide having the sequence NGQLAPGVYIVTFSA and therefore represents the epitope of $V_HH#2$.

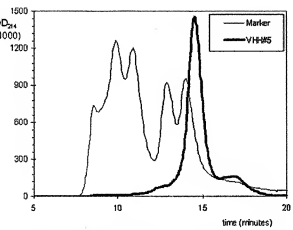

FIG. 6. Size exclusion chromatography of V_HH#5. V_HH#5 and a mixture of marker proteins were analyzed on a Superdex 75 column, and the measured OD_{214} was plotted against the retention time. The peaks of the marker proteins (13.7, 25, 43, 67, and 2,000 kDa) are shown.

most probably due to the strong interaction between the unusual long CDR3 regions of heavy-chain antibodies and functional centers of proteins (9, 27). As has been found with enzyme inhibiting heavy-chain antibodies, the extended CDR3 loops might protrude into clefts or cavities present on the surface of viral or phage particles and block them (48, 49). A large panel of different p2-specific V_HH fragments was selected from a phage display library and with a specially developed assay, a limited number of phage-neutralizing antibodies were identified.

Sequence analysis revealed that all the antibody fragments examined have the characteristics of the heavy-chain variable region of single-domain antibodies (47). The four analyzed ELISA-positive, but nonneutralizing, V_HHs contain completely different sequences, and in particular, the variability seems to be localized in the CDRs. The length of the CDR3 is highly diverse, varying from 5 residues for V_HH#2 to 15 residues for V_HH#1 and V_HH#4. Antibodies V_HH#2 and

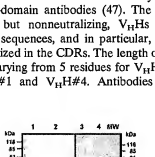

FIG. 7. Specificity of neutralizing V_HH#5 determined by Western blot analysis. Phage sk1 was loaded (lane 1, 6×10^9 PFU; lane 2, 3×10^{10} PFU) onto a 15% gel, blotted, and incubated with V_HH#5. The other part of the gel (lane 3, 1×10^{10} PFU; lane 4, 2×10^{10} PFU) was stained with Coomassie brilliant blue (CBB). The experimentally determined amino-terminal sequence (ThrIleLysAsnPheThrPhePheSer ProAsnSerThrGluPhe; the expected methionine as start residue has been clipped off) of the recognized protein (arrows) corresponds to the hypothetical gene product of ORF18. MW, molecular mass.

V_HH#3, which both react with the msp of p2, are also entirely different in sequence, suggesting that both fragments might recognize different epitopes. Indeed V_HH#2 recognizes an epitope shared by phages p2 and sk1, while V_HH#3 reacts with a distinct antigenic site, which exclusively occurs within the mcp of phage p2. Alignment of the amino acid sequences using previously published data (25) revealed that only four amino acid differences exist between the msp of phage p2 and sk1. Two of these changes are separated by only nine residues, thereby making this region a prominent candidate for the epitope of V_HH#3. This area is located 15 residues upstream of the epitope for V_HH#2.

The three analyzed neutralizing antibodies V_HH#5, V_HH#6, and V_HH#7 are less variable in sequence and have a CDR3 of the same length (14 amino acids), with only a few differing residues within this important region. This indicates that this group of antibodies recognizes the same antigen and probably also an identical epitope.

The phage-inhibiting capacity of the antibody fragment turned out to be dependent on the titer of the phage contamination. When titers of 10^5 to 10^5 PFU/ml were tested, which in a production plant would result in failed fermentation, an antibody concentration as low as 2.25 nM gave complete neutralization. This corresponds well to the measured affinity (K_D = 1.40 nM) of the antibody fragment. This means that the proportion of antigen not bound by antibodies must be rather high. This high efficiency may be explained in terms of competition between antibody (with a high affinity for the phage) and cell-bound receptor (with a lower affinity) for binding to phage particles. The antibody concentration used, 2 nM, corresponds to 10^{12} molecules/ml. From Western blot data, we estimate that 10 copies of ORF18 protein are present per phage particle, which implies 10^4 to 10^6 ORF18 molecules per ml, while 10^{12} V_HH molecules are present with a high affinity (1.4 nM) for ORF18, whereas only about 10^{10} ORF18 receptor molecules are present per ml of bacterial cultures, with a moderate affinity for ORF18 (a few hundred nanomolar).

The antibody fragment prevents infection with the only other 936-like phage examined (sk1) but failed to neutralize two members of the c2 group, which is the second most prevalent lactococcal phage group and is genetically distinct from the 936-like phages. This indicates that V_HH#5 will only protect against 936-like phages. The efficacy of fragment V_HH#5 was evaluated in cheese production experiments using a 200-liter vessel (28). The results were in line with those described for the small-scale cultures; addition of V_HH#5 to a final concentration of 7 or 70 nM completely neutralized phage infections of 10^3 to 10^5 PFU/ml, and acidification profiles identical to those of noninfected control were obtained. Using the above-determined values for interactions and lysis and a set of differential equations, we mimicked cell lysis by phages in the presence and absence of V_HH#5 and ORF18 at various concentrations of *L. lactis* cells. Depending on the number of ORF18 proteins per phage cell, lysis can be predicted reasonably well (data not shown).

It was demonstrated that antibody fragment V_HH#5 recognizes the ORF18-encoded gene product, for which no function was known (4). The antigen is a constituent of the tip of the phage tail, as shown by immunoelectron microscopic studies. FASTA analysis with the amino acid sequence of ORF18 gave

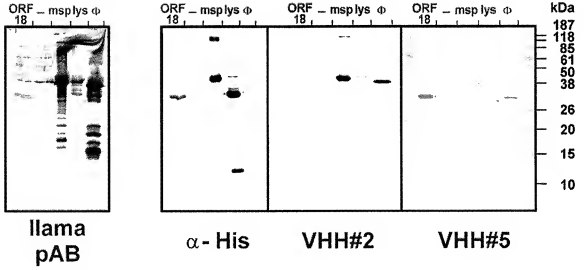

FIG. 8. Western blot analysis with phage p2-derived proteins expressed in *E. coli* or complete bacteriophage particles of sk1. The antibody fragments V_HH#2 and V_HH#5 and postimmune llama serum (llama pAB blot) were used for detection. Purified ORF18, msp, lysin (lys), and a bacterial extract of *E. coli* cells containing the expression vector used (−) and phage sk1 (Φ) were loaded. The hexahistidine-tagged proteins were detected with anti-His monoclonal antibody (α-His).

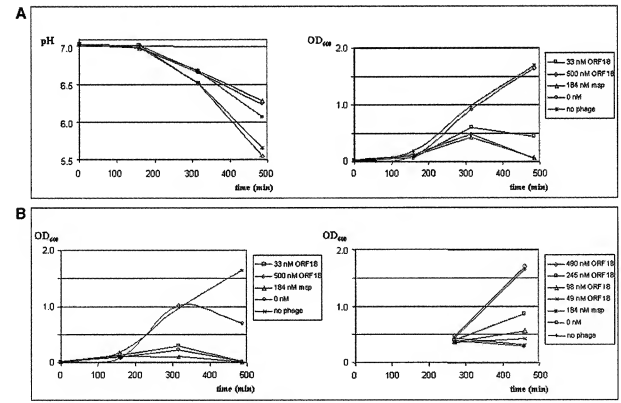

FIG. 9. Effect of ORF18 protein on infection as measured by acidification and the kinetics of growth. (A) Two concentrations of ORF18 were included in cultures of *L. lactis* C2 containing 10^2 PFU/ml bacteriophage p2. Infected and noninfected cultures were used as controls, while purified msp was added to another infected culture. (B) Partial protection by addition of ORF18 against higher phage titers (10^5 PFU/ml) as measured by the kinetics of growth (graph on the left). The dose-dependent neutralizing effect of ORF18 on cultures with phage titers of 10^2 PFU/ml was studied in more detail with a new batch of purified ORF18 (growth curves on the right).

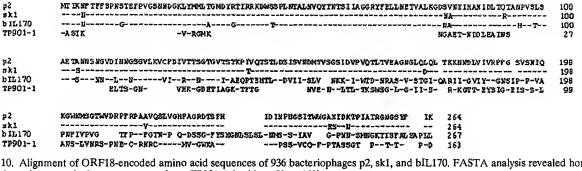

FIG. 10. Alignment of ORF18-encoded amino acid sequences of 936 bacteriophages p2, sk1, and bIL170. FASTA analysis revealed homology with the baseplate protein from temperate phage TP901-1 (residues 53 to 163).

the expected hits with the corresponding gene product of phage sk1 and the gene 120 protein from *Lactococcus* phage bIL170, which both belong to the 936 group.

Interestingly, some similarity was found with the baseplate protein (*orf bpp*) from the temperate lactococcal bacteriophage TP901-1, a P335-like phage (38) (Fig. 10). The homology with *orf bpp* was found within the carboxy-terminal domain of the gene product, of which the localization within the phage particle was established by immunogold labeling (22). Comparison of the late regions of phages sk1 and lambda by gene size and the predicted isoelectric points of their ORFs (4) previously hinted toward a relationship of ORF18 with phage lambda gene K, which belongs to the cluster of tail genes.

This conclusion is in agreement with the proposed function of *orf bpp* from phage TP901-1. Knocking out almost the complete gene of *orf bpp* in the lysogenic phage resulted in the production of noninfectious phage particles (38). The authors mentioned the possibility that this might be achieved by binding of *orf bpp* directly to the cells or indirectly by binding with other proteins involved in the phage-host interaction. However, our competition experiments strongly suggest a direct interaction.

The *L. lactis*-encoded receptor (*pip*) for c2-like phages has been identified (7, 12–14) and was shown not to be responsible for the entrance of 936-like (11) or P335-like (24) phages. During FASTA analysis, no homology of ORF18 of phage p2 was found with genes from phage c2 (29). Since phage c2 binds to another type of receptor, it can be assumed that the receptor-binding proteins of phage c2 and p2 are different, explaining why V_HH#5 does not neutralize c2 infections. As a potential function of ORF18 has now been elucidated, this protein can be used for the characterization of a *Lactococcus* receptor, which is most likely to be a cell surface glycoprotein.

During this study, we have also proven that camelid antibodies can be raised against proteins of a complete biological entity, which opens the opportunity to develop protein arrays based on these antibodies (26). Our electron microscopy studies showed that V_HHs can be used to study protein complexes in biological systems. Moreover, based on the knowledge gained and the technologies developed in this study, the development of V_HHs that neutralize other prokaryotic or eukaryotic viruses can be envisioned.

ACKNOWLEDGMENTS

We are grateful to S. Muyldermans (Vrije Universiteit Brussels, Belgium) for useful suggestions concerning the experimental work. We are grateful to N. van Riel (Technical University of Eindhoven) for developing a model for cell lysis and to D. Tremblay and L. Boucher for technical assistance in lactococcal phage purification. We thank W. Bos, C. Bulkmans, J. van Heemskerk, P. Hermans, I. Schaffers, and C. van Vliet for technical assistance and J. Chapman, M. van Egmond, and M. van der Vaart, all from Unilever Research Laboratories Vlaardingen, for discussions.

S. Moineau was supported by the Natural Sciences and Engineering Research Council of Canada.

ADDENDUM

Following acceptance of the manuscript, Dupont et al. (K. Dupont, F. K. Vogensen, H. Neve, J. Bresciani, and J. Josephsen, Appl. Environ. Microbiol. **70:** 5818–5828, 2004) also showed that ORF18 is the receptor-binding protein of 936-like lactococcal phages.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 265, No. 18, Issue of June 25, pp. 10347-10351, 1990
Printed in U.S.A.

Properties of Monoclonal Antibodies Selected for Probing the Conformation of Wild Type and Mutant Forms of the P22 Tailspike Endorhamnosidase*

(Received for publication, February 14, 1990)

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Eleven species of monoclonal antibodies directed against the trimeric P22 tailspike endorhamnosidase have been selected and characterized. Seven of these antibodies recognize the native tailspike, both isolated and assembled onto the virion, and prevent phage infection. Four antibodies react with denatured forms of the tailspike as well as with the plastic adsorbed tailspike. Three of these latter prevent the tailspike from assembling onto the phage head.

The antibodies have been tested against tailspike proteins carrying single amino acid substitutions at 15 different sites on the protein. Two of these mutations interfere with binding by a set of the monoclonals, indicating that they disrupt the epitopes for these antibodies. Since amino acid replacements corresponding to the temperature-sensitive folding mutations do not change the conformation of the native protein, these mutant proteins may be particularly useful for mapping epitopes.

Amber fragments of the tailspike chain are recognized predominantly by the anti-denatured antibodies suggesting either that they are conformationally closer to folding intermediates than to the native tailspike or that the epitopes recognized by anti-native antibodies are carried by the C-terminal end of the native protein. Immunochemical detection by an anti-denatured antibody, after sucrose gradient sedimentation of a large 55-kDa amber fragment, indicates a monomeric rather than a trimeric state. This suggests that the missing C-terminal region is important for the trimerization reaction. Such N-terminal amber fragments may be useful models for studying with the monoclonal antibodies the nascent chain emerging from the ribosome.

The P22 tailspike is a trimer, composed of three 71.6-kDa polypeptide chains encoded by gene 9 of the phage (1–3). Each trimeric spike is asymmetric and elongated, about 220 Å by about 60 Å. The secondary structure is dominated by β sheet/turn conformations (4). In the course of particle assembly, six tailspikes bind to a unique vertex of the phage heads forming the cell attachment apparatus. The tailspikes have an endorhamnosidase activity which cleaves the 0 antigen projecting off the *Salmonella* cell surface (5).

Native tailspikes are thermostable, requiring temperatures of 88 °C for heat denaturation (6), resistant to proteases, and to denaturation by detergent. As a result two relatively long lived intracellular intermediates in the chain folding and association can be distinguished from the native protein (7, 8). After release from the ribosome, newly synthesized chains form an early single chain intermediate. These mature to a species capable of chain association. The product, the protrimer, is a metastable species which can be trapped in the cold (7). The chains in the protrimer are associated but not fully folded. This species matures into the thermostable, detergent and protease resistant, native tailspike.

The tailspike has been the subject of a genetic analysis of intracellular chain folding and association (9–11). A large set of temperature-sensitive folding mutants has been isolated and characterized. These mutants destabilize the early single chain intermediate and block the pathway prior to the protrimer stage. They presumably identify sequences involved in directing the conformation of folding intermediates. Many of these sites are hydrophilic and located at the surface of the mature molecule (12).

We are interested in the possibility of characterizing folding intermediates, and relating the conformation of the intermediates to the conformation of the native protein, using monoclonal antibodies. The experience with β_2 subunit of tryptophan synthase indicated that this would be possible using the native protein as antigen (13–16). Therefore, we have selected monoclonal antibodies raised with native tailspikes and characterized their target specificity. The results reported indicate essentially that some antibodies can recognize conformational precursors and may be useful probes to investigate conformational changes upon folding and assembly of the tailspike polypeptide chains.

EXPERIMENTAL PROCEDURES[1]

RESULTS AND DISCUSSION

Anti-native and Anti-denatured Protein Recognition—The ability of 11 monoclonal antibodies to recognize the native

* This work was supported by Grant URA D1129 from the Centre National de la Recherche Scientifique and by the Université Paris VII and the Fondation pour la Recherche Médicale. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] Portions of this paper (including "Experimental Procedures," Tables I–III, and Fig. 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE IV

Recognition by 11 monoclonal antibodies of the coated tailspike, the tailspike, and the amber fragments in solution

Monoclonal antibodies	Reactivity of the tailspike		Absorbance in the competition test[a]		Amber fragments recognition[b]		
	Coated tailspike	Coated tailspike heat-denatured	Without the tailspike	With the tailspike	55 and 52 kDa	46 kDa	23 and 14 kDa
33–2	+	−	0.45	0.20	−	−	−
51–2	+	−	0.85	0.10	−	−	−
54–1	+	−	0.40	0.30	−	−	−
155–3	+	−	1.00	0.30	−	−	−
175–3	+	−	1.20	0.05	±	±	−
219–2	+	−	1.20	0.05	−	−	−
236–3	+	−	1.00	0.05	±	−	−
70–5	+	+	0.50	0.45 (0.35)	+	+	+
92–3	+	+	1.20	1.00 (0.80)	+	−	−
105–3	+	+	0.40	0.40 (0.35)	+	−	−
124–5	+	+	0.40	0.30 (0.05)	+	−	−

[a] Absorbance values obtained in the ELISA competition test (see "Experimental Procedures") after a 30-min or overnight incubation (in brackets) of the antibodies without or with the tailspike in solution.

[b] After 15 h of incubation in the ELISA competition test.

P22 tailspike protein was tested by using an ELISA[2] competition test in solution to eliminate problems of adsorption-associated denaturation (24). The results obtained are reported in Table IV. An ELISA screening test was also done with both the coated tailspike and the coated and then heat-denatured tailspike to select antibodies recognizing denatured forms of the protein (see "Experimental Procedures"). Four antibodies (70–5, 92–3, 105–3, and 124–5) were found to recognize well the coated tailspike, the coated heat-denatured tailspike, but not to recognize the native antigen in solution after 30 min of incubation (anti-denatured antibodies) (Table IV). The lack of reactivity of the four anti-denatured antibodies with the soluble tailspike, although they recognize well the coated form, shows once again that the protein may undergo, at least partially, denaturation upon adsorbing to the ELISA plate (24, 28). Seven antibodies reacted with the tailspike coated or in solution but not with the coated heat-denatured protein (anti-native antibodies). The weak reactivity in solution of the antibody 54–1 can be explained by its low affinity (10^7 M^{-1}) for the tailspike. An alternative explanation could be that this antibody reacts with a hidden epitope of the native protein which is exposed upon the coating but is disrupted upon the heat denaturation.

The equilibrium dissociation constant of the antibodies for the native tailspike was determined in solution (25) and is reported in Table IV. For the four anti-denatured antibodies, the antibody-antigen complex formation might proceed by a previous spontaneous unfolding, at least locally, of the native protein in solution allowing the exposure of hidden epitopes and then the fixation of the antibody. Thus, the apparent affinity value obtained is probably not the true equilibrium constant due to these two coupled equilibria.

Effect of the Monoclonal Antibodies on the Binding of Tailspikes to Head—One of the properties of the mature tailspike is its ability to bind noncovalently but irreversibly to phage heads, converting them to infectious virions (1, 27). If any of the tailspike epitopes were at or near the site(s) needed for joining to the heads, incubation with antibodies might interfere with the tailing reaction. None of the anti-native antibodies (33–2, 51–2, 54–1, 155–3, 175–3, 219–2, and 236–3) inhibited the head tailing since no free heads were recovered (see Tables II and V). However, three of the anti-denatured antibodies (92–3, 105–3, and 124–5) inhibited the tailing reaction, while antibody 70–5 did not.

Effect of Monoclonal Antibodies on Cell Killing by the Phage—The tailspikes carry out their normal functions of cell attachment and infection while bound to the DNA injection vertex of the phage particle. To determine if the antibodies inhibit cell killing by the phages, the viability of *S. typhimurium* cell was measured in the presence of phages preincubated with or without monoclonal antibodies. As shown in Tables III and V, only the antibodies defined as anti-native (33–2, 51–2, 54–1, 155–3, 175–3, 219–2, and 236–3) prevented phage infection. The anti-denatured antibodies (70–5, 92–3, 105–3, and 124–5) did not. Incubation of the antibodies with cells in the absence of phage had no effect on cell viability.

The observation that the anti-native antibodies inhibit cell killing indicates that they must be binding to the tailspike when it is bound to the virion. Thus, the epitopes for these seven antibodies are on the surface of the spike that is accessible to the antibodies in the virion. Consistent with this observation is the failure of the anti-native antibodies to prevent tailspikes from binding to heads: as they do not interfere with the head binding (Table V) they might recognize exposed surfaces of the tailspikes in the virion.

The four anti-denatured antibodies did not prevent the phages from killing *Salmonella* cells. Either they bound to sites distant from the active site without inhibiting the endorhamnosidase activity or they were unable to bind to the tailspike when it is likely incorporated into the virion. As shown above, three of the four species interfered with the binding of free tailspike to phage head. This could suggest that the sites of recognition are in, or close to, the regions of the protein buried in the particle assembly reaction; but as these antibodies recognize the denatured form of the protein, it is more likely that they bind and trap the tailspike in a conformation unable to react with the head.

Reactivity of the Monoclonal Antibodies with Different tsf Mutant Proteins—The reactivity of the monoclonal antibodies against the native purified tailspike protein from 15 *tsf* mutants (see Table I under "Experimental Procedures") was determined by using first a classical indirect ELISA with coated antigens. Thirteen out of the 15 mutant proteins were recognized by the different monoclonal antibodies as well as the wild type. The two others: *tsfH304* (Gly^{344} → Arg) and *tsfH302* (Gly^{323} → Asp) did not react with some monoclonal antibodies. This result was confirmed with an ELISA competition test where either the wild-type protein, the *tsfH304* mutant protein or the *tsfH302* mutant protein were incubated

[2] The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; *tsf*, temperature-sensitive folding; SDS, sodium dodecyl sulfate.

TABLE V

Antibody classification emerging from some discriminatory properties

Monoclonal antibodies[a]	Native tailspike recognition[b] and cell killing inhibition	Tail-head assembly inhibition	23- and 14-kDa amber fragments recognition	$Gly^{244} \rightarrow Arg$ mutant protein recognition	$Gly^{323} \rightarrow Asp$ mutant protein recognition	K_d[c]	Class
						mol/liter	
33-2	+	−	−	−	+	3×10^{-9}	I
54-1	+	−	−	−	−	1×10^{-7}	II
51-2	+	−	−	−	−	3×10^{-10}	
175-3	+	−	−	−	−	1×10^{-10}	III
219-2	+	−	−	−	−	5×10^{-11}	
236-3	+	−	−	−	−	1×10^{-10}	
155-3	+	−	−	+	+	2×10^{-9}	IV
70-5	−	−	+	+	+	6×10^{-8}	V
92-3	−	+	−	+	+	2×10^{-7}	
105-3	−	+	−	+	+	6×10^{-7}	VI
124-5	−	+	−	+	+	2×10^{-9}	VII

[a] Antibodies 70-5, 92-3, 105-3, and 124-5 recognize the coated heat-denatured tailspike and were referred as anti-denatured antibodies.

[b] After 30 min of incubation in the ELISA competition test.

[c] Dissociation equilibrium constant obtained with the tailspike in solution by the method of Friguet *et al.* (25).

(either 30 min or overnight) in solution with the antibodies (Table V).

That all the monoclonal antibodies recognized 13 out of the 15 mutant proteins tested conforms to the view that the native forms of the mutant proteins have conformations very close to that of the wild type (4, 12). Since the $Gly^{323} \rightarrow Asp$ mutant was recognized by monoclonal antibody 33-2 (class I) (see Table V) but not by monoclonal antibodies 51-2, 54-1, 175-3, 219-2, and 236-3 (classes II and III), the epitopes of class I on one hand and classes II and III on the other hand are clearly different. The failure of the $Gly^{244} \rightarrow Arg$ mutant to be recognized by monoclonals from class I as well as from classes II and III suggests that this residue could be at or near the corresponding epitopes, however, the change of Gly^{244} by Arg could induce changes in the conformation of these epitopes.

Recognition of Different Amber Fragments by the Antibodies—The conformational and association state of amber fragments of the tailspike chain had not been yet determined. The immunoreactivity of five amber fragments ranging in size from 14 to 55 kDa was examined using cell lysates containing different amber fragments (see proteins under "Experimental Procedures") or the wild-type tailspike protein. The experiments with the coated proteins showed on the one hand that the wild-type tailspike in the coated lysate was recognized by the antibodies as well as the pure coated wild-type tailspike. On the other hand, the anti-native antibodies (33-2, 51-2, 54-1, 155-3, 175-3, 219-2, and 236-3) did not recognize the coated amber fragments while the anti-denatured antibodies (70-5, 92-3, 105-3, and 124-5) recognized amber fragments 23, 46, 52, and 55 kDa.

The experiments with the proteins in solution showed (Table IV) that three of the anti-denatured antibodies reacted only with two large amber fragments (52 and 55 kDa). The fourth anti-denatured antibody (70-5) recognized the five amber fragments indicating that its epitope is present in the N-terminal 14-kDa amber fragment.

With two anti-native antibodies, a weak reactivity was found with the biggest fragments in solution: antibody 175-3 with 55-, 52-, and 46-kDa fragments and antibody 236-3 with 55- and 52-kDa fragments (Table IV). This result indicates either that the epitopes (or part of the epitopes) recognized by the five other anti-native antibodies are located on the C-terminal end of the protein, or that the amber fragment carries the epitope in a different conformation than in the whole protein. In the latter hypothesis, the anti-native antibodies might be useful probes to investigate the folding of the polypeptide chains during their release from the ribosome as single chain, their assembly within the protrimer intermediate and the native trimeric spike, since they would react differently with the newly synthesized protein in these different states.

Evidence for a Monomeric Form of 55-kDa Amber Fragment—To determine if the 55-kDa amber fragment contained in the cell lysate is in a monomeric or a trimeric form, a sucrose gradient centrifugation was performed and the amber fragment was detected by an anti-denatured antibody (92-3). As controls and references, purified tailspike protein, tailspike protein contained in the cell lysate, and commercially available pure carbonic anhydrase and serum albumin were centrifuged in sucrose gradient under the same experimental conditions (Fig. 1) (see "Experimental Procedures"). The native tailspike protein ($M_r = 3 \times 72$, 9.3 *s*) gave the same profile either in its purified form or included in the cell lysate. The 55-kDa amber fragment sedimented between carbonic anhydrase (2.8 *s*) and bovine serum albumin (4.3 *s*) (see *inset* of Fig. 1) giving an estimate of 3.1 *s* for the sedimentation coefficient. This result indicates that the 55-kDa fragment is not associated into a trimer but is monomeric and that the C-terminal part could be involved in the chain association reaction.

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Supplementary Material To:

Properties of Monoclonal Antibodies Selected for Probing the Conformation of Wild Type and Mutant Forms of the P22 Tailspike endorhamnosidase.

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EXPERIMENTAL PROCEDURES

Bacteria and Bacteriophages

All bacterial strains are derivatives of *Salmonella typhimurium* LT2. Strain DB7155 (hsdS 20(gal)) was the permissive host for phage carrying amber mutations while the restrictive host was DB7136 (*1*7).

The gene *9* amber mutants *amN110*, *amH120*, *amE1017*, *amH1014*, *amH703* was described by Smith *et al.* (*18*). The phage carries contained, in addition to the amber mutation in gene *9*, amber mutations in gene *3* (*amN114*) and in gene *13* (*am101*). When grown on the restrictive (*su*$^-$) host, the gene *3* mutation prevents the assembly of the capsids while the gene *13* mutation delays cell lysis past the normal time. All phage strains also carry the clear plaque cI-7 allele to ensure entry into the lytic cycle of phage growth.

Phages without tails (heads) were prepared as described (*1*).

Media

The media were those described (*18-19*). The M9 minimal medium was supplemented with 0.002% (w/v) L-histidine, 0.0015% (w/v) L-histidine and 0.01% (w/v) yeast extract. A 0.1% (w/v) tryptone and 0.7% (w/v) NaCl solution was used as dilution fluid.

Proteins

Highly purified P22 tailspike proteins from wild type and mutants (Table I) were obtained as previously described (*9*, *11*, *20*). Wild type tailspike protein and amber fragments were produced after phage infection of *S.typhimurium* DB7136. The preparation of infected cells and cell lysates was done as described (*20*). Briefly cell lysates containing amber fragments or the wild type tailspike protein were prepared with *S.typhimurium* DB7136 (suppressor negative host) infected by phage strains carrying gene *9* mutations: *amN110*, *amH120*, *amE1017*, *amH1014*, *amH703* and *s*$^+$/*13*$^-$. The production of the corresponding fragments (14 kDa, 23 kDa, 46 kDa, 52 kDa and 55 kDa) and the complete tailspike chain (72 kDa) was checked by electrophoresis of each crude extract on a 12.5% polyacrylamide gel in the presence of SDS. The concentration of the fragments was estimated by comparative staining densitometry of the gel, to be at least 0.1 mg/ml. The ability of the amber fragments or the tailspike, contained in the lysates, to be recognized by the antibodies was first tested by an indirect ELISA where a 100-fold dilution of the lysates was used onto the nitrocellulose plate.

To analyze the recognition of the fragments in solution, an ELISA competition test was performed where a serial dilution of each lysate was incubated with the antibodies at 0.4 pg/ml for 15h at 4°C.

Monoclonal Antibody Preparation

BALB/c mice were immunized as previously described (*21*) with purified native P22 wild type tailspike protein. The hybridomas production was done as described (*22*). The hybridoma culture supernatants were screened for the presence of antibodies by the enzyme linked immunosorbent assays described below (see ELISA techniques). Out of the 180 populations obtained, 33 were found to produce antibodies recognizing either the coated tailspike (i.e. the tailspike immobilized on the ELISA plate) or the coated heat-denatured protein (i.e. the immobilized tailspike denatured by brazing the coated ELISA plate at 100°C for 5 min). The positive populations were grown on a large scale for storage in liquid nitrogen and 11 of them were selected and cloned. The cloning was done by limit dilution. The cloned hybridomas were ascitized and injected intraperitoneally into pristane-primed BALB/c mice. The immunoglobulin fraction of the ascitic fluid was purified by ammonium sulfate precipitation (40% saturation) followed by an ion exchange chromatography as described by Friguet *et al.* (*23*). The antibody solutions were stored in phosphate buffered saline (PBS) pH 7.2 at -20°C.

ELISA Techniques

Both the classical indirect ELISA and the ELISA competition test were used (*24*). To select also antibodies specific for a denatured epitope of the P22 tailspike protein, the ELISA plates were coated with P22 tailspike protein, sealed with adhesive tape and incubated for 5 min in boiling water to induce an irreversible thermal denaturation of the coated protein.

The affinity of the different antibodies for the tailspike protein was measured by the method of Friguet *et al.* (*25*).

Isotype Determination

The class specificity on the 11 monoclonal antibodies was determined using a classical indirect ELISA with tailspike as the coated antigen and class-specific antibodies linked to horse radish peroxidase. Except antibody 54-1 which is IgM, all the antibodies are IgG.

Binding of Tailspike to Head

Initial experiments were performed to determine the incubation time and tailspike concentration required for efficient tailing of the heads. 5×10^8 heads/ml were tailed with 5×10^{-8} M tailspike protein after a 30 min-incubation at 30°C. Reconstitution of viable phage from heads and tailspikes was followed by counting the phage plaques on a *S.typhimurium* lawn after a 10^6-fold dilution of the test suspensions. Since some antibodies prevent phage infection of the bacteria (see below), it was important to discriminate between the nontailed heads and the inactive antibody-phage complexes. For this purpose, the following protocol was used:

Step 1: Each monoclonal antibody (25 µl at 4×10^{-6} M) was mixed with tailspike protein (25 µl at 10^{-7} M) and then incubated for 15 h at 4°C. To determine the 100% tailing level, 25 µl PBS instead of antibody was mixed with tailspike protein at the same concentration and incubated in the same conditions.

Step 2: The preincubated mixtures of antibody and tailspike were added to 50 µl of heads at 10^9 heads/ml and incubated for 30 min at 30°C.

Step 3: To eliminate the free antibody (and eventually the free antibody-tailspike complexes), 25 µl polyethyleneglycol 6000 at 30% (w/v) in NaCl 2.5 M was added and the samples were centrifuged at 4°C for 75 min at 15,000 x *g*. The supernatants were removed and the pellets suspended in 50 µl M9 supplemented with 1 mM $MgSO_4$. The samples were filtered to measure the formation of phage (Table II).

Step 4: To detect the presence of nontailed (free) heads in the suspended pellets, 50 µl of additional free tailspike protein at 10^{-7} M were added and incubated for 30 min at 30°C. The resulting active phages were counted and compared with the titer before this step.

The addition of free tailspikes does not displace antibodies from antibody-phage complexes when these complexes are present in the pellet. Indeed, when the same protocol (as described above) from step 3 was applied to P22 phages (5×10^8/ml) previously incubated for 15h at 4°C with 2×10^{-6} M of each inactivating antibody, no additional active phages were recovered in the presence of tailspike.

Cell Killing by the Phage

P22 phages (0.2 ml at 1.7×10^9 phages/ml) were mixed with each monoclonal antibody (0.2 ml at 4×10^{-6} M) and incubated for 15h at 4°C. For the controls, either the phages (same volume and concentration) were incubated with 0.2 ml PBS (100% killing control) or the antibodies (same volume and concentration) were incubated with 0.2 ml M9 supplemented with 1 mM $MgSO_4$ (0% killing control). Each of these preincubated mixtures was added to *S.typhimurium* cells (0.4 ml at 4×10^8 cells/ml), harvested in exponential phase, and incubated for 10 min at 30°C. Then, the cell suspensions were diluted by a factor 10^6 and viable cells counted by plating on Luria Broth plates (Table III). The cell density was 8.4×10^8/ml± 15%.

Gel Electrophoresis and Protein Measurements

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed either with a slab gel (26) or using the Phast system from Pharmacia.

An estimate of the amber fragments or the tailspike concentration in cell lysates was done after staining of electrophoresis gels with Coomassie Blue using samples containing known amounts of tailspike protein as standards and a LKB 2202 Ultroscan densitometer coupled with a LKB 2220 integrator. Purified protein concentrations were determined by absorbance measurements. The absorption coefficients used were 1.5 $mg^{-1}.ml.cm^{-1}$ and 0.98 $mg^{-1}.ml.cm^{-1}$ at 280 nm for monoclonal antibodies and P22 tailspike protein respectively (3).

Sucrose Gradient Sedimentation

Sucrose gradient sedimentation was performed using a SW50.1 rotor-equipped Beckman L M-6 ultracentrifuge. Linear sucrose gradients (4.5 ml 5% to 20% (w/v) in 62.5 mM Tris-HCl pH 6.8) were poured into the tubes. Underneath these gradients were layered, first, 200 µl of 50% (w/v) CsCl in 62.5 mM Tris-HCl pH 6.8. Then 200 µl of each cell lysate to be tested were mixed with 5 µl of carbonic anhydrase and bovine serum albumin at 10 mg/ml, loaded on top of the gradients and then centrifuged at 45,000*xg* for 20h at 4°C. Gradients were fractionated into constant volume fractions from a pinhole at the bottom of the tube. Fractions of 0.25 ml were collected and analyzed both by SDS gel electrophoresis and ELISA for the recognition by the antibody 92-3. A 20-fold dilution of each fraction was used for the coating of the ELISA plate. The absorbances obtained after the enzymatic reaction for the different fractions are reported in Fig. 1.

Table I

Purified native forms of mutant tailspikes carrying *tsf* amino acid substitutions

Mutant	Substitution[a]	Mobility change[b]
tsf U34	Lys^{163} -> Glu	yes
tsf U55	Glu^{190} -> Lys	yes
tsf U57	Asp^{230} -> Val	yes
tsf U2	Asp^{238} -> Ser	yes
tsf H304	Gly^{264} -> Arg	yes
tsf U24	Ile^{258} -> Leu	no
tsf RH	Val^{270} -> Gly	no
tsf U19	Arg^{285} -> Lys	yes
tsf U18	Thr^{307} -> Ala	no
tsf MU9	Glu^{309} -> Val	yes
tsf H302	Gly^{323} -> Asp	yes
tsf H08	Glu^{344} -> Lys	yes
tsf U14	Glu^{405} -> Lys	yes
tsf M9	Glu^{364} -> Lys Arg^{285} -> Lys Asp^{230} -> Asn	yes
tsf U58	unknown	yes

[a] Villafane & King (11) and Yu & King (12).

[b] Yu & King (12).

Table II

Effect of monoclonal antibodies on the assembly of tailspikes onto the head

Antibodies	Active phages (x 10^8 ml)	
	Before step 4[a]	After step 4[a]
None	4.6	5.4
33-2	0.0	0.0
51-2	0.1	0.1
54-1	0.2	0.6
155-3	0.0	0.0
175-3	0.0	0.0
21-92	0.2	0.1
236-3	0.1	0.0
70-5	4.8	4.9
92-3	0.0	4.5
105-3	0.0	5.6
124-5	0.0	5.6

[a] As described in the Experimental Procedures section, in step 4 the heads which remained free after the incubation of the heads and antibody-tailspike complexes are detected by the addition of tailspike in the absence of free antibody. The difference between the number of active phages after and before step 4 gives the number of heads remaining free after incubation with the tail-antibody mixture.

Table III

Effect of the monoclonal antibodies on the cell[a] killing activity of P22 phages[b]

Antibodies	Cells (x 10^8/ml)		% cell killing
	In absence of phage	In presence of phage	
None	8.0	3.1	61
33-2	8.0	9.8	0
51-2	7.5	9.6	0
54-1	8.0	9.6	0
155-3	8.4	9.0	0
175-3	8.4	8.4	0
219-2	8.3	8.4	0
236-3	8.3	8.4	0
70-5	8.6	4.2	51
92-3	7.8	3.9	50
105-3	7.2	3.9	46
124-5	8.7	3.3	62

[a] *S.typhimurium* DB7136.

[b] *cI-13-amH101113-amN114.*

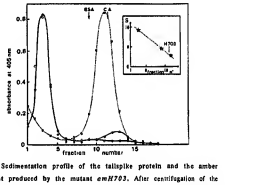

FIG. 1 Sedimentation profile of the tailspike protein and the amber fragment produced by the mutant *amH703*. After centrifugation of the proteins through the sucrose gradient (see Experimental Procedures), fractions of 0.25 ml were collected from the bottom to the top and tested for their reactivity with the antibody 92-3 by an indirect ELISA test. The absorbances obtained in the ELISA test are plotted versus the fraction number: o—o, pure tailspike protein; •—•, cell lysate containing the tailspike protein; ▪—▪, cell lysate containing the amber fragment of the mutant *amH703*.

The arrows indicate the peak of the fractions containing the marker proteins carbonic anhydrase (CA) and bovine serum albumin (BSA) detected by SDS-polyacrylamide gel electrophoresis.

Inset of Fig. 1: The S values of the marker proteins carbonic anhydrase (2.8 S), bovine serum albumin (4.3 S) and the native tailspike protein (9.3 S) are plotted versus the fraction number. The fraction containing the amber fragment is indicated by the arrow.